

Studies of rabbit testes infected with *Treponema pallidum*

II Local synthesis of antibodies

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SUMMARY Samples of serum and testicular fluid from normal rabbits and those infected with *Treponema pallidum* were examined for total protein, albumin, IgG, and IgM concentrations. Total protein concentrations were similar in testicular fluid from normal and infected animals but were lower than in serum. The concentrations of individual proteins in serum did not differ substantially between infected and non-infected rabbits. In testicular fluid, however, the concentrations of all three proteins, especially IgM, were greater in infected rabbits. A significantly ($p < 0.02$) raised testis-IgM index, together with a higher concentration of anti-treponemal IgM in the infected testicular fluid than in autologous serum, suggests local synthesis of IgM antibodies.

Introduction

Natural and experimental syphilis is characterised by humoral response to *T pallidum* specific and non-specific treponemal antigens. Although antibodies are synthesised by plasma cells, and these have been repeatedly observed in syphilitic lesions,¹⁻³ no information is available regarding their role at the site of infection. In this report we demonstrate local synthesis of antibodies in rabbit testes infected with *T pallidum*, confirm the presence of plasma cells in the lymphocytic infiltration in the testes, and provide evidence that one of the functional roles of plasma cells at the site of infection is the production of treponemal antibodies.

Materials and methods

PREPARATION OF SAMPLES OF TESTICULAR FLUID AND SERUM

Each of 11 Nys (Flemish Giant) rabbits was infected with 20×10^6 *T pallidum* organisms (Nichols strain)

per testis. Orchitis developed between days 9 and 12 after infection. Blood was taken from the heart and the animals killed.³ The testes were removed aseptically, cut into halves, and centrifuged at $10\,000 \times g$ for one hour. Depending on the degree of infiltration, 1 to 2 ml of infected testicular fluid was obtained. Testes of eight normal rabbits, processed similarly, yielded about 0.2 ml of normal testicular fluid per testis. Samples of serum and normal and infected testicular fluid were stored in aliquots at -65°C .

ANTISERA

Unlabelled and fluorescein isothiocyanate labelled goat IgG antibodies specific for rabbit γ and μ chains, goat anti rabbit albumin, and rabbit anti goat IgG antibodies were obtained from Cappel Laboratories, Cochranville, Pennsylvania, United States.

TECHNIQUES

Double diffusion gel precipitation tests were performed in Petri dishes with 1.5% Noble agar (Difco Laboratory, Detroit, Michigan, United States) using the Ouchterlony method.⁴ Immunoelectrophoresis was performed with LKB equipment (LKB Instruments, Rockville, Maryland, United States) and barbital buffer (pH 8.6, ionic strength 0.05) using the Scheidegger method.⁵ Total protein concen-

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trations in serum and testicular fluid were measured by the Lowry method.⁶

The single radial immunodiffusion test was used to measure IgG, IgM, and albumin in serum and testicular fluid.⁷ Agar containing antibody was prepared by using 0.1 ml of specific antiserum in 15 ml of 1.5% agarose (Marine Colloids Division, FMC Corporation, Rockland, Maryland, United States) in phosphate buffered saline (PBS). Wells 3 mm in diameter were cut and filled with 5 μ l of the material examined. The plates were incubated for various periods at 4°C. Three dilutions of the serum and testicular fluid from a single animal were examined simultaneously in the same plate, which also contained various concentrations of an appropriate standard.

Standard IgG was prepared in our laboratory from pooled normal rabbit serum by using a diethylaminoethanol (DEAE) cellulose fraction eluted with 0.005 mol/l phosphate buffer. Standard IgM was obtained from a pool of serum samples from 20 normal rabbits (1 ml per rabbit). Based on our experience with immunoglobulin determination in rabbits, we arbitrarily assigned to the pool a concentration of IgM 1 mg/ml. Because of the low concentration of IgM in the pool it was concentrated five times in a B-15 Minicon concentrator (Amicon Corporation, Lexington, Massachusetts, United States). The concentrate (IgM 5 mg/ml) and dilutions of it were used as a standard, as was crystallised albumin (Alb, Sigma Chemical Company, St Louis, Missouri, United States).

The diameters of precipitation rings for IgG and albumin were measured after incubation for 18-24 hours. No ring was visible for IgM regardless of the antigen to antibody ratio or the incubation time. After incubation for 48 hours at 4°C the IgM plates were washed for two days at 4°C in PBS with several daily changes. Precipitation rings then developed with rabbit anti goat IgG after an additional incubation period of 18-24 hours. A standard curve was prepared for each protein and the concentrations of IgG, IgM, and albumin in the samples of serum and testicular fluid were extrapolated.

IDENTIFICATION OF TREPONEMAL ANTIBODIES BY FLUORESCENCE TESTS

Samples of serum obtained before infection and of serum and testicular fluid obtained from six animals at the peak of orchitis were examined for total production of antitreponemal antibodies (specific and cross reacting) and determination of the class(es) of immunoglobulins involved. Quantitative fluorescent treponemal antibody (FTA) tests (without sorbent) and FTA-ABS tests (in the presence of sorbent) were carried out with slides containing electrophoretically purified *T pallidum* (Beckman Instruments, Fullerton, California, United States). Fluorescein isothiocyanate labelled goat IgG antibodies to rabbit γ or μ chain were used as conjugates (Cappel Laboratories). The volume of reagents, incubation conditions, and washing times were as described.⁸

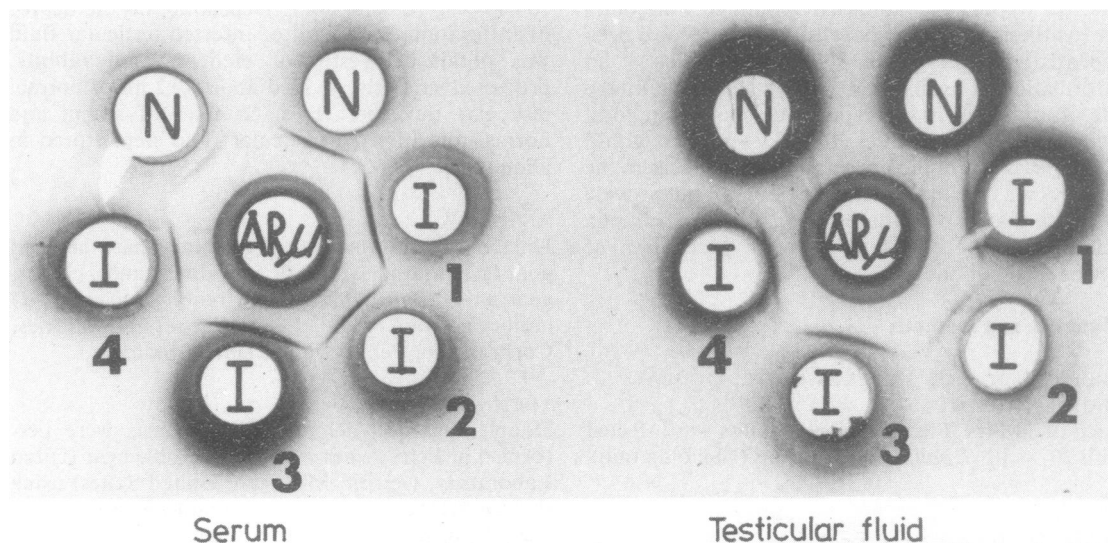


FIG 1 Double diffusion gel precipitation tests of samples of serum and testicular fluid from four infected (I) and two normal (N) rabbits with goat anti-rabbit IgM serum (AR μ).

Results

Antisera to rabbit γ and μ chains were examined in our laboratory by immunoelectrophoresis with undiluted whole normal rabbit serum. One precipitation line formed with each antiserum. The antiserum to rabbit IgM was also examined by the two step single radial immunodiffusion test with purified IgG and globulin depleted rabbit serum as antigens, the latter kindly provided by Cappel Laboratories. The lack of precipitation ring indicated that the antiserum did not contain antibodies to light chains or to macroglobulins other than IgM.

Serum samples of eight normal animals and 11 infected with *T. pallidum* contained similar concentrations of IgM detectable by double diffusion gel precipitation tests (as shown in Fig 1). In samples of testicular fluid, however, a difference was obvious; those from infected animals showed a definite precipitation line after incubation for 48 hours, whereas in those from normal rabbits the precipitation line (close to the antigen well) was hardly visible, even after staining.

PROTEIN CONCENTRATIONS

The table shows that total protein concentrations were similar in the serum of normal and infected animals but varied slightly in the samples of testicular fluid. The concentrations were always lower in the testicular fluid than in the corresponding serum. Each sample of serum and testicular fluid was examined by single radial immunodiffusion test for albumin, IgG, and IgM concentrations at three dilutions. No significant differences were found between serum from infected and normal rabbits. In infected testicular fluid, however, all protein concentrations, especially that of IgM, were raised.

Several formulas are helpful for distinguishing among the possible mechanisms that could account for raised immunoglobulin concentrations in various organs or fluids. The simplest, the ratio of immunoglobulin to total protein concentrations, is applicable when only IgG concentration is increased. For

infected testicular fluid, in which both immunoglobulins and total protein concentrations are raised, the most suitable formula is the testis immunoglobulin index:

$$\frac{\text{Testicular fluid Ig/serum Ig}}{\text{Testicular fluid albumin/serum albumin}} \times 100\%$$

(immunoglobulin and albumin measured in mg/ml)

Figure 2 shows that the mean (SD) index was slightly less (59% (19%) v 67% (30%)) for IgG and appreciably more (101% (30%) v 65% (15%)) for IgM in infected v normal rabbits. The difference between IgM means was significant by Student's *t* test ($p < 0.025$) and by the non-parametric Mann-Whitney U test ($p < 0.015$).

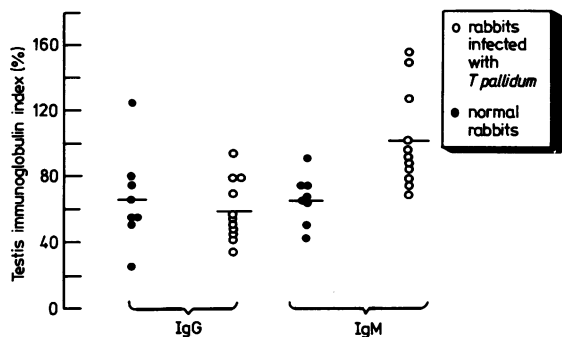


FIG 2 Testis immunoglobulin index measuring local synthesis of immunoglobulin in testicular fluid from eight normal and 11 infected rabbits. Bars represent means.

TREPONEMAL ANTIBODIES

Serum samples taken before infection and tested without sorbent (FTA test) demonstrated low titres ($\leq 1/10$) of antitreponemal IgM and IgG, both of which were removed by the sorbent (FTA-ABS test) (fig 3). Samples of serum and testicular fluid obtained 9 to 12 days after infection showed a substantial increase in titres of total antitreponemal

TABLE Mean (SEM) protein concentration in three dilutions of serum and testicular fluid in normal and *T. pallidum* infected rabbits

Protein	Infected rabbits (n = 11)		Normal rabbits (n = 8)		Ratio of infected to normal:	
	Serum*	Testicular fluid*	Serum	Testicular fluid	Serum	Testicular fluid
Total protein	49.45 (2.69)	38.96 (0.87)	47.83 (1.05)	31.50 (0.88)	1.03	1.24
Albumin	28.35 (9.12)	25.40 (1.69)	32.35 (1.58)	14.82 (0.73)	0.88	1.71
IgG	6.36 (0.52)	3.22 (0.18)	5.76 (1.93)	1.59 (0.09)	1.10	2.03
IgM	1.21 (0.19)	0.94 (0.11)	0.99 (0.05)	0.29 (0.03)	1.22	3.24

*Samples taken at peak of orchitis (9-12 days).

IgM, which was higher in testicular fluid (mean (SD) titre 1/173 (1/118)) than in serum (1/147 (1/32)). In contrast the increase in total antitreponemal IgG concentration was smaller in testicular fluid (1/43 (1/20)) than in serum (1/86 (1/39)). In all tests sorbent removed more than half of the total antibodies.

Discussion

Total proteins in testicular fluid consist of specific testicular proteins and serum proteins which result from a transudation from blood across a normal blood-testis barrier. Some proteins similar to serum proteins might also be locally produced by Sertoli's cells,⁹ even though there is no indication that they are immunoglobulin in nature. Despite the multiplicity of sources, the concentration of total proteins is lower in testicular fluid than in serum. Immuno-electrophoretic examination of testicular fluid with anti whole rabbit serum shows a pattern of a diluted serum in which IgG and albumin are always visible but IgM is present in only small amounts or not at all.

In infected testicular fluid total and individual protein concentrations are higher, although the increase is not the same for all proteins. In this study the ratios in infected to normal testicular fluid were 1.24 for total protein, 1.71 for albumin, 2.03 for IgG, and 3.24 for IgM. These results suggested that, in addition to an increased leakage of serum proteins by exudation across an altered blood-testis barrier, some immunoglobulins must be synthesised locally.

Several findings support this assumption. In the first paper in this series we demonstrated by experiments with cell surface markers and mitogenic stimulation that plasma cells are among the first cells attracted to the site of inoculation.³ Furthermore, specific treponemal antibodies were detected in testicular fluid before they could be detected in serum. In this study, with specific anti isotype antibodies, the mean (SD) testicular fluid immunoglobulin indexes for infected ν normal rabbits were 59% (19%) ν 67% (30%) for IgG and 101% (30%) ν 65% (15%) for IgM. An increased exudate could not explain these results, particularly as the IgM molecule is about five times larger than that of IgG and should therefore have more difficulty in crossing the blood-testis barrier.

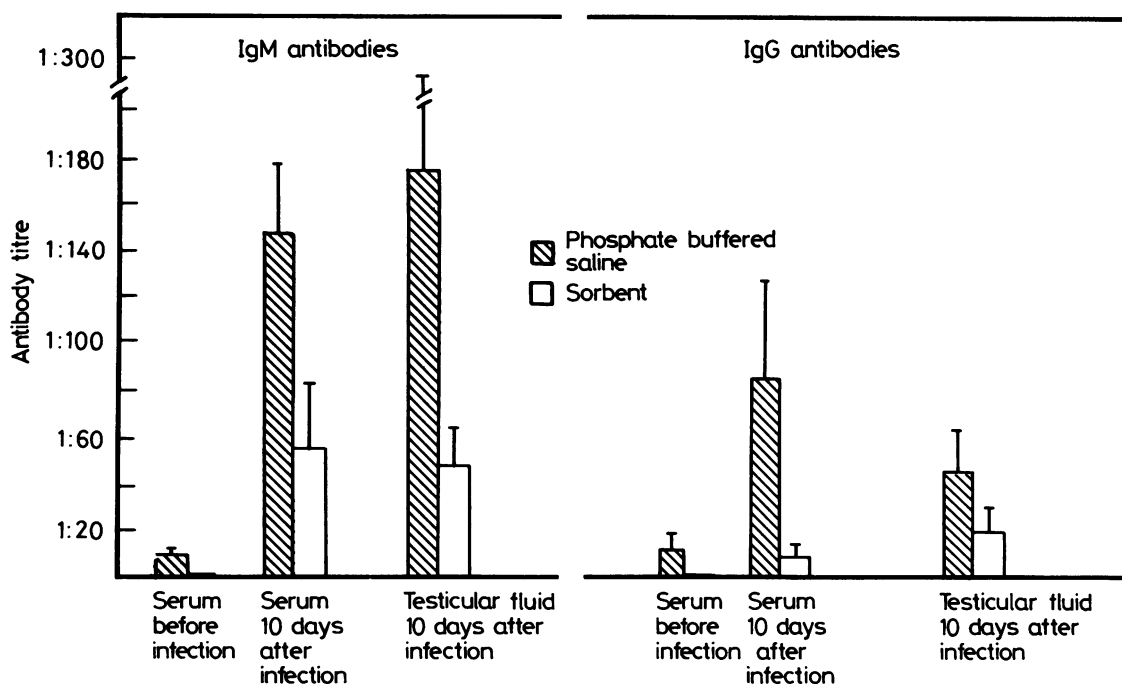


FIG 3 Antitreponemal antibodies in serum before infection with *T pallidum* and in samples of serum and testicular fluid during orchitis (10 days after infection). Bars represent standard deviations.

Local synthesis of IgM seems to be a more plausible explanation for the increase but, if the increase is relevant to syphilitic infection, it must be correlated with an increase in treponemal antibodies. Since rabbits infected with *T. pallidum* produce both specific and cross-reacting antitreponemal antibodies, local immunoglobulin production should parallel total antitreponemal antibody synthesis. Concentrations of total IgM antibody were much higher than those of total IgG antibody in serum and in testicular fluid in infected animals. While it may be argued that differences of one or two dilutions in IgM titres are not statistically significant, the consistency of the findings obtained in this study by two different procedures strongly supports our assumption.

Although a local increase in IgM concentrations, particularly at the early stage of the immune response, is not surprising, it is more difficult to explain why IgG concentrations are lower in testicular fluids than in serum. If plasma cells in the testes have the same immunological competence as those in extratesticular lymphoid organs, one would expect the same concentration of IgG in testicular fluid as in serum, since IgG could originate either from serum exudate or from the testicular plasma cells. A possible explanation for the lower IgG concentrations in testicular fluid is that plasma cells in the testes are not as immunologically competent,

or are under a selective suppressive effect, or both. We report strong immunosuppressive activity by testicular cells and lymphocytic infiltrates¹⁰; the relevance of this finding to the course of syphilitic infection needs to be clarified.

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